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The osteogenic properties of CaP/silk composite scaffolds

Yufeng Zhang^{1,2†}, Chengtie Wu^{2†}, Thor Friis², Yin Xiao^{*1,2}

¹ Ministry Education Key Laboratory for Oral Biomedical Engineering School of Stomatology, Wuhan University, Wuhan 430079, P. R. China

² Institute of Health & Biomedical Innovation, Queensland University of Technology, Brisbane, Queensland 4059, Australia.

[†] Authors contributing equally to the work.

* Author to whom correspondence should be addressed.

E-mail: yin.xiao@qut.edu.au

Ph: 61-7-31386240

Abstract

The rationale for the present study was to develop porous CaP/silk composite scaffolds with an optimized CaP-phase distribution and pore architecture, to facilitate osteogenic properties of human bone mesenchymal stromal cells (BMSCs) and *in vivo* bone formation abilities. This was achieved by first preparing CaP/silk hybrid powders which were then incorporated into silk to obtain uniform CaP/silk composite scaffolds, by means of a freeze drying method. The composition, microstructure and mechanical properties of the CaP/silk composite scaffolds were analyzed by X-ray diffraction (XRD), Fourier transform infrared spectra (FTIR), scanning electron microscope (SEM) and a universal mechanical testing machine. BMSCs were cultured in these scaffolds and cell proliferation analyzed by confocal microscopy and MTS assay. Alkaline phosphatase (ALP) activity and osteogenic gene expression were assayed to determine if osteogenic differentiation had taken place. A calvarial defect model in SCID mice was used to determine the *in vivo* bone forming ability of the hybrid CaP/silk scaffolds. Our results showed that incorporating the hybrid CaP/silk powders into silk scaffolds improved both pore structure architecture and distribution of CaP powders in the composite scaffolds. By incorporating the CaP phase into silk scaffolds *in vitro* osteogenic differentiation of BMSCs was enhanced and there was increased *in vivo* cancellous bone formation. Here we report a novel method to prepare Ca/P composite scaffolds with an optimum pore structure and Ca/P distribution to facilitate BMSC differentiation and bone formation.

Introduction

Silk fibroin has been widely trialed in biomaterial studies in the form of silk fibers [1-3], membrane [4], microsphere [5] and porous scaffolds [6-9], by virtue of its combination of mechanical properties, controllable biodegradability and cytocompatibility [10, 11]. As a novel bone tissue engineering material, three-dimensional (3D) porous silk scaffolds are receiving more attention because of their excellent physiochemical properties [7, 12-14] and much progress has been made in understanding how cells interact with silk scaffolds [15, 16]. The osteo-conductivity of silk scaffolds has been improved by coating apatite on the surface of silk scaffolds using a precipitation method. The mechanical strength of the scaffolds, however, tends to be impaired by this method [17]. Previous studies have mainly prepared the 3D silk scaffolds by a salt-leaching method which has tended to result in compromised interconnectivity and pore structure [10]. The present study therefore sought to develop novel Ca/P composite scaffolds with pore structures, interconnectivity, and mechanical properties more suited for bone tissue engineering.

One of most challenging issues when preparing inorganic/organic scaffolds is how to uniformly distribute the inorganic powders within the matrix of polymers [18]. Inorganic powders have a tendency to aggregate easily due to electrostatic interaction, which in turn leads to a suboptimal pore structure and distribution of inorganic materials within the scaffolds. The surface property of the inorganic powders is an

important factor which influences the powder distribution and pore structure of composite scaffolds. In this study, we first prepared CaP/silk hybrid powders, which were then incorporated into silk solution, to fabricate uniform CaP/silk composite scaffolds by a freeze drying method. In the process we developed a new method to prepare porous CaP/silk composite scaffolds with potential applications in bone regeneration.

2. Materials and Methods

2.1. Preparation and characterization of CaP/silk hybrid powders

CaP/silk hybrid powders were prepared using a co-precipitation method. Typically, 0.374 g of $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$ (Sigma Aldrich, Castle Hill, NSW, Australia) and 2 g of silk solution (5 % w/v) was dissolved in 24 mL deionized water and 0.588 g of $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ (Sigma Aldrich) was dissolved in 40 mL ethanol. Silk protein fibroin was extracted from mulberry silk cocoons following a standard extraction procedures [19]. The NaH_2PO_4 and silk water solution was added to CaCl_2 ethanol solution drop by drop under stirring. The deposited hybrid powders were filtered, washed three times with water and once with ethanol, then vacuum-dried at 40 °C for one day. Pure Ca/P powders without silk were prepared under the same preparation conditions and used for controls.

The CaP/silk hybrid powders thus prepared were characterized by X-ray diffraction (XRD), scanning electron microscope (SEM) and Fourier transform infrared spectra (FTIR).

2.2. Preparation, characterization and mechanical strength of CaP/silk porous composite scaffolds

Porous silk scaffolds containing CaP/silk hybrid powders were fabricated using a freeze-drying method. Briefly, CaP/silk hybrid powder, at a final concentration of either 5 or 10% (w/v), was added into 10 mL of 5% (w/v) silk water solutions under stirring to form a uniform mixture. The mixture was transferred into plastic dishes at 4 °C for 30 min, and then placed in a freezer at -35 °C overnight to solidify the solvent

and induce solid–liquid phase separation. The solidified mixture was maintained at -80°C for 2 hr and was freeze-dried in a freeze-drying vessel (OHRIST BETA 1-15, Germany) for 48 hr after which they were treated with 90% (v/v) ethanol. These scaffolds will be referred to as “hybrid CaP/silk” scaffolds. The silk scaffolds containing pure CaP powders were prepared as control by the same method and are referred to as “CaP/silk scaffolds”. Pure silk scaffolds were also prepared for controls.

The pore morphology and microstructure of the top surface, cross section and bottom surface of the scaffolds, were characterized by SEM.

The compressive strength and modulus of the scaffolds (10mm×10mm×10mm) were measured using a universal testing machine (Instron) at a crosshead speed of 0.5 mm/min.

2.3. Cell morphology and proliferation on CaP/silk scaffolds

Scaffolds were placed in 96-well plastic culture plates and incubated in Dulbecco’s modified Eagle media (DMEM; Invitrogen, Mt Waverley, VIC Australia) overnight. The media was aspirated off and then 1×10^5 BMSCs in a 50 μ L suspension volume was placed on each scaffold. The cells were allowed to adhere to the scaffolds for 3 hr before the cell–scaffold complexes were covered with 150 μ L of culture medium (DMEM + 10% FBS). Cell viability and proliferation was determined using CellTiter 96 Aqueous One Solution Reagent (Promega, Genesearch. QLD, Australia). Briefly, cells were incubated at 37°C in 5% CO₂ for 1, 7 and 14 days, at which point 100 μ L of 0.5 mg/mL MTS solution was added to each well and incubated for 4 hr at

37°C. Formazan absorbance was read at 490 nm using a plate reader and software Accent/MTS. After 7 day incubation, the scaffolds were fixed in 2.5% glutaraldehyde for scanning electron microscope (SEM) and confocal microscopy.

2.4. Alkaline phosphatase (ALP) activity of BMSCs

Osteogenic differentiation was assessed by measuring alkaline phosphatase (ALP) activity of BMSCs grown on the various scaffold types. Scaffolds were cut into 5×5×1 mm pieces and transferred into 24-well plastic culture plates and a total of 1×10⁵ BMSCs were seeded onto each scaffold. After 1 day the medium was changed to osteogenic differentiation medium (high glucose DMEM containing 10% FBS, 50 µM ascorbic acid 2-phosphate, 10 mM β-glycerol phosphate and 100 nM dexamethasone (Sigma Aldrich)), which was changed every 4 days. On days 7 and 14 samples were removed and ALP activity was measured. The scaffold were first irrigated three times with PBS, to remove as much residual serum as possible, and then 1 mL of 0.02% Triton® X-100 were placed on the scaffolds which dissolved the cells. The solution was transferred into a 1.5 mL tube, and sonicated and then the samples were centrifuged at 14,000 rpm at 4°C for 15 min, and the supernatant transferred to fresh 1.5 mL tubes to which 100 µL 1 M Tris-HCl, 20 µL 5 mM MgCl₂, and 20 µL 5 mM p-nitrophenyl phosphate was added. After incubation for 30 min at 37°C the reaction was stopped by the addition of 50 µL of 1N NaOH. Using p-nitrophenol as a standard, the absorbance was measured at 410 nm with a spectrophotometer. The ALP activity was expressed as the value of p-nitrophenol production quantity divided by the reaction time and the total protein synthesis

quantity measured by a BCA Protein assay kit (Thermo Scientific, Melbourne, Australia).

2.5. Reverse transcription and real-time quantitative RT-PCR analysis

The effect of Ca/P silk scaffolds on BMSC osteogenic differentiation was further assessed by real-time quantitative RT-PCR (RT-qPCR) to measure the mRNA expression of ALP, type I collagen (COL1) and osteocalcin (OCN) in all treatment groups (Table 1). Scaffolds were cut into 8×8×2 mm pieces and transferred into 24-well plastic culture plates and a total of 10⁶ BMSCs were placed onto each scaffold. The medium was changed after 24 hours to osteogenic differentiation medium. On day 14 the samples were removed and total RNA isolated using Tri-Reagent® (Sigma Aldrich) according to the manufacturer's instructions. Complementary DNA was synthesized from 1 µg of total RNA using SuperScript III (Invitrogen) following the manufacturer's instructions. RT-qPCR was performed in 25 µL reaction volume containing 12.5 µL 2X SYBR Green Master Mix (Roche, Castle Hill, NSW, Australia), 2.5 µL each of 10 µM forward and reverse primers, 2.5 µL of cDNA template diluted 1:10, and 5 µL of RNase free water. All samples were performed in triplicates and the house keeping gene, 18s rRNA, was used as a control. The reaction was carried out using ABI Prism 7000 Sequence Detection System (Applied Biosystems) and the PCR amplification followed 1 cycle of 10 min at 95°C, 40 cycles of 15 s at 95 and 60°C for 1 min. Melting curve analysis was performed to validate specific amplicon amplification without genomic DNA contamination. Relative expression levels for each gene were normalized against the Ct value of the

house keeping gene and determined by using the delta Ct method. The relative expression of each gene was analysed by one-way ANOVA and Student–Newman–Keuls (SNK) q-test. The significant difference was considered at $P<0.05$.

2.6. Transplantation into calvarial defects

The bone forming ability of different scaffolds were assessed in a calvarial defect model in severe combined immunodeficient (SCID) mice [20]. Pure silk and 5% hybrid CaP/silk scaffolds were cut into $3\times3\times1$ mm pieces, transferred into 24-well plastic culture plates, and a total of 1×10^5 BMSCs was seeded on each scaffold. The medium was changed the following day with osteogenic differentiation medium. The medium was changed every 4 days and on day 14 the complex was implanted into the bone defect.

The surgeries were carried out according to the guidelines of the Animal Research and Care Committee of the Herston Medical Centre and Queensland University of Technology. The surgical procedures were performed in aseptic conditions under general anesthesia. Briefly, a 10 mm linear incision was made on the left side of the skull to reveal the bone surface. The periosteum was dissected from the bone surface and a full-thickness calvarial bone defect, 3 mm in diameter, was created with a trephine bur with a slow-speed dental drill. To prevent heating injuries to the animals, 0.9% physiological saline was dropped onto the contact point between the bur and bone and care was taken to avoid injury to the dura of the animals. The implant was trimmed to fit the defect and placed precisely into the defects, and soft

tissue above the defect was closed with skin staples. Control sites included calvarial defects in which no implants were placed.

Animals were euthanized 4 weeks after surgery and the defect areas were collected. All samples were scanned for bone formation within the defect after implant retrieval with a μ CT40 imaging system (Scanco Medical, Bassersdorf, Switzerland) with the following scan parameters: 20 mm field of view, 55 kVp x-ray energy setting, 1024 reconstruction matrix, slice thickness 0.02 mm, and a 250 millisecond integration time. Mineralized tissue was segmented from non-mineralized tissue using a global thresholding procedure with a value approximating 1.20 g/cm³ (150 on μ CT) (25% lower than 1.6 g/cm³ which is the mineral density of healthy human compact bone. Bone volume per defect (BV; mm³) was recorded as the measure of defect bone regeneration.

Following the μ CT scan, the samples were fixed in 4% paraformaldehyde for 12 hr at room temperature and then decalcified in 10% EDTA, which was changed twice weekly, for 2 to 3 weeks before being embedded in paraffin. Serial sections of 5 μ m were cut and mounted on polylysine-coated slides, and all sections were stained with hematoxylin and eosin for general assessment of the tissue and wound healing. Specimens were examined with light microscope.

2.7. Statistical analysis

All experiments were performed in triplicate, with each treatment also conducted in triplicate. Means and standard deviations (SD) were calculated, and the statistical significance of differences among each group was examined by one-way ANOVA and

a post hoc t-test. Significance was set at $p < 0.05$ level.

3. Results

3.1. Characterization of CaP/silk hybrid powders and composite scaffolds

Figure 1 shows the XRD patterns of the pure CaP powders and CaP/silk hybrid powders. The main phase composition for pure CaP powders and CaP/silk hybrid powders is monetite ($\text{CaPO}_3(\text{OH})$) (JCPD card No: 09-0080). FTIR analysis confirmed the formed CaP/silk hybrid powders by the existence of P-O characteristic peaks at the wavenumbers of 562 and 1080cm^{-1} , and silk characteristic peaks at the wavenumbers of 1500 and 1600 cm^{-1} (Fig. 2). SEM analysis showed that the prepared CaP/hybrid powders were composed of the lathlike particles with a diameter of 500 nm and the sheet-like particles with an approximate size of $3\text{ }\mu\text{m}$ (Fig. 3b). Pure CaP powders were composed of irregular particles with an approximate size of 1-3 μm (Fig. 3a).

Pure silk and silk composite scaffolds with high porosity were prepared by a freeze-drying method. Pore structure and distribution was analyzed in the top surface and inner microstructures of pure silk scaffolds and composite scaffolds. This analysis revealed that pore structure and distribution was more uniform in hybrid-CaP/silk scaffolds than in CaP/silk scaffolds (Fig. 4). The bottom surface of the scaffolds showed no obvious aggregation of CaP powders in the hybrid CaP/silk scaffolds (Fig. 5b and c), whereas CaP powders in CaP/silk scaffolds became aggregated (see arrows) (Fig. 5d and e). The incorporation 5% and 10% of CaP powders into silk scaffolds did not influence the compressive strength and compressive modulus of the scaffolds (Fig. 6).

After having established that hybrid CaP/silk scaffolds had improved pore structure and powder distribution compared to the CaP/silk scaffolds, our focus shifted to the hybrid scaffolds for the subsequent experiments, which included mechanical testing, BMSCs attachment, proliferation, differentiation and in vivo experiments.

3.2. BMSCs morphology, proliferation and ALP activity

After 1 day of culture, BMSCs were found to have attached and were distributed even to the inner of scaffolds with a spread out morphology (Fig. 6 a, d, g). After seven days the number of cells had increased significantly in all three types of scaffolds. There was no noticeable structural or cell number difference among the three types of scaffolds (Fig. 7 b, e, h), and confocal microscopy images showed that cells were uniformly distributed within the scaffolds (Fig. 7 c, f, i).

There was a clear temporal proliferation profile of the BMSCs after 1, 7 and 14 days of culture on all three scaffold species (Fig. 8a), but no obvious proliferation differences between scaffold species (Fig. 8a).

All three scaffold species supported BMSCs differentiation and the 5% and 10% hybrid CaP/silk scaffolds showed a significantly higher ALP activity level after 7 and 14 days of culture compared to pure silk scaffolds (Fig. 8b). There was, however, no obvious difference between the 5% and 10% hybrid CaP/silk scaffolds.

3.3. Real time quantitative RT-PCR

The osteoblastic differentiation was further assessed by RT-qPCR of ALP, COL1 and OCN mRNA expression. There was a significant upregulation of the osteogenic marker genes ALP, COL1 and OCN in the BMSCs after 2 weeks in osteogenic

culture media in the hybrid CaP/silk scaffolds compared to those in pure silk scaffolds (Fig. 9).

3.4 *In vivo study*

Quantification of the mineralized areas in skull bone defects showed only low levels of calcified tissue in either the unfilled defects or defects filled with pure silk scaffolds only (Fig 10). There was a significant increase of calcified tissues in the defects filled with CaP/silk scaffold compared to both unfilled defects and defects filled with pure silk scaffolds. Interestingly, incorporation of BMSCs with hybrid CaP/silk scaffolds induced the most significant tissue calcification in bone defects ($p < 0.05$).

There was no histological evidence of an inflammatory reaction in any of the treatment groups four weeks after implantation (Fig. 11). Histological analysis confirmed the bone formation that was observed in the micro-CT scans. Unfilled calvarial defects were covered with a thin fibrous connective tissue sheet with no evidence of new bone formation. Bone defects filled with silk fibroin scaffold alone showed increased amounts of cells and fibrous tissue within the defects, however, no new bone was observed. New bone formation was found in defects filled with Cap/silk scaffold, silk and hybrid CaP/silk scaffolds seeded with BMSCs. In pure silk scaffolds seeded with BMSCs, newly formed bone was only formed in areas adjacent to the rim of the defects. In defects filled with CaP/silk scaffolds (both CaP/silk and hybrid CaP/silk), with and without BMSCs, bone islands were identified both peripherally and centrally within the scaffolds. In addition to this, mineralized

bony trabeculae were also found and these were surrounded by osteoblasts.

4. Discussion

In this study we incorporated hybrid CaP/silk powders into silk scaffolds to improve the pore structure, architecture and distribution of CaP powders in the composite scaffolds. This approach resulted in enhanced *in vitro* osteogenic differentiation of BMSCs and new bone formation scattered inside the scaffolds in a skull bone defect model.

Inorganic/organic scaffolds have traditionally been prepared by direct incorporation of inorganic powders into polymer materials. Distributing the inorganic powders uniformly throughout the matrix of polymers is the greatest challenge associated with this method, primarily due to aggregation of the powders and incompatible surface properties between the inorganic materials and polymers [21, 22]. In this study, we have applied a novel method to produce a hybrid of CaP/silk powders by the co-precipitation of CaP particles and silk. The hybrid CaP/silk powders had an improved interface compatibility with the silk scaffolds as well as improved of CaP distribution within the silk scaffolds, which was reflected in significantly less aggregation of CaP particles on the silk surfaces.

CaP/silk scaffolds can be prepared by coating a layer of CaP on the surface of a silk scaffold using a precipitation methods [17], in which the silk scaffolds is first prepared by a salt-leaching method. Although the CaP/silk composite scaffolds produced by this method have improved osteoconductive properties, these composite

scaffolds do not have interconnecting pores, and the mechanical strength of the scaffolds is weakened when covered with CaP particles. In the method we applied, hybrid-CaP/silk powders were incorporated into silk scaffolds by freeze-drying. The scaffolds produced this way had far better interconnectivity and an uniform distribution of CaP particles, and this was achieved without compromising the mechanical strength of the scaffolds[17].

Scaffolds porosity is important for aiding cell functions, by providing a foundation for cell attachment, proliferation, and cell matrix formation [23, 24]. The porous CaP/silk scaffolds appeared to provide a good environment for BMSC growth and differentiation. There was a near 4-fold increase in the number of cells on the CaP/silk scaffold after two weeks of culture, and in the presence of osteogenic media BMSCs showed increased expression levels of osteogenic markers. It seems quite possible the influence of CaP particles on BMSC differentiation is the reason for the better osteogenic differentiation properties of the CaP/silk scaffold compared with the pure silk scaffolds alone. Calcium phosphate containing materials, when hydrated in cell culture media, undergo a process of continuous calcium and phosphate ion dissolution and reprecipitation, and this process can induce osteoblast differentiation and mineralization [23]. Furthermore, CaP particles are known to enhance the healing of bone defect by stimulating osteoblast differentiation [25].

The fundamental premise of tissue engineering is regeneration of tissues and restoration of organ function through implantation of cells grown outside the body or by stimulating cells to migrate into an implanted matrix. [26] The clinical success of

the construct depends to a large degree on the quality of the scaffold and supplied cells. It is our view that the material we have developed may be used to accelerate new bone formation in bone defects. The *in vivo* study showed that pure silk scaffolds did not induce any bone formation, in spite of these materials having an excellent pore structure. The hybrid CaP/silk scaffolds, on the other hand, with a pore structure similar to that of pure silk scaffolds, did induce bone formation. Also, the amount of bone induced by BMSCs seeded on hybrid CaP/silk scaffolds was significantly greater than the amount of bone induced by BMSCs on silk scaffolds. There are a couple of likely factors contributing to this improved bone forming ability: firstly, the hybrid CaP/silk scaffolds had a more uniform distribution of CaP particles which had the effect of enhancing bone cell differentiation and further improved bone formation after implantation into the bone defects. The second reason is that the hybrid CaP/silk scaffolds had a more interconnected and larger pore structure, and this was beneficial for cell ingrowth and nutrient supply and providing a more optimum environment for new bone formation.

5. Conclusions

Our results demonstrated that incorporation of hybrid CaP/silk powders into silk scaffolds improved the porous structure and distribution of CaP powders in the composite scaffolds, which enhanced *in vitro* BMSCs osteogenic differentiation, and also improved *in vivo* bone formation. This study proposed a novel method to fabricate hybrid CaP/silk scaffolds for potential cell based therapy in bone defect repair.

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